Journal of Environmental Science and Health

Part A
Environmental Science
and
Engineering
including
Toxic and Hazardous Substances Control

Part B
Pesticides, Food Contaminants
and
Agricultural Waste

Part C
Environmental Carcinogenesis Reviews

Journal of Environmental Science and Health
Part A
ENVIRONMENTAL SCIENCE AND ENGINEERING
Including Toxic and Hazardous Substances Control

Part B
PESTICIDES, FOOD CONTAMINANTS, AND AGRICULTURAL WASTES

Part C
ENVIRONMENTAL CARCINOGENESIS REVIEWS

J. ENVIRON. SCI. HEALTH, A27(3), 663-695 (1992)

BIODEGRADATION OF 2,4- AND 2,6-DINITROTOLUENE BY FRESHWATER MICROORGANISMS

Key Words: 2,4-Dinitrotoluene, 2,6-Dinitrotoluene, Microbial degradation, Mineralization

Howard T. Bausum, Wayne R. Mitchell, and Michael A. Major

United States Army Biomedical Research and Development Laboratory
Health Effects Research Division
Fort Detrick, Frederick, Maryland 21702 U.S.A.

ABSTRACT

The microbial degradation of 2,4- and 2,6-dinitrotoluene was complete or nearly complete in surface water from two locations downstream from the Radford Army Ammunition Plant. No degradation was detected in surface water from four local (Frederick, MD area) sites. Either isomer could serve as a sole carbon and energy source, with about 60 percent of substrate carbon appearing as CO_2, and with an increase in the population of degrading organisms. In both the rate of mineralization in percent degraded per day increased with increasing substrate concentration. At

10 mg/L, degradation rates of 32 and 14.5 percent/day were observed for the 2,4 and 2,6 isomers, respectively. At very low concentrations of the 2,6 isomer a degrading population did not develop, and significant degradation did not occur. The rate of substrate utilization was far greater, and the lag time shorter, for the 2,4 isomer, consistent with a far greater density of 2,4-DNT degraders.

Mixed enrichment cultures were developed for each DNT isomer separately, by sequential transfer to increasing substrate concentrations. Maximum substrate concentrations utilized were about 130 mg/L, and cell yields of 6.8 to 7.3 X 10^5 CFU/µg input DNT were calculated. Disappearance of 2,4-DNT in the presence of high concentrations of 2,4-DNT mixed enrichment culture approximated first-order kinetics; pseudo-first order rate constants varied from 0.043 to 0.190 min^{-1}. The mean second-order constant was 3.9 X 10^{-10} ml $cell^{-1}$ min^{-1}. If one assumes a concentration of 10^6 cells/ml, at 25°C, a half-life of 29.7 hours can be estimated for this isomer. Similarly, for 2,6-DNT, the second-order constant was 9.9 X 10^{-10} ml $cell^{-1}$ min^{-1}. The corresponding estimated half-life was 11.6 hours. This rate is not likely to be realized in natural surface waters, due at least in part to the very low densities of 2,6-DNT utilizers. From mixed enrichment cultures, pure cultures using 2,4-DNT were isolated.

INTRODUCTION

Dinitrotoluene (DNT) occurs in six isomeric forms, of which 2,4-DNT and 2,6-DNT are the most important[1] in terms of quantities produced. The general properties and uses of these two compounds have been reviewed by Etnier[2]. Dinitrotoluene is used as an intermediate in the manufacture of toluene diisocyanate and thence polyurethanes. It also finds application in the manufacture of dyes and as an ingredient in both military and commercial explosives. A somewhat purified form of 2,4-DNT is used in smokeless powders. DNT is prepared by the nitration of toluene or nitrotoluene in the presence of nitric and sulfuric acids.

The DNT's are also produced as by-products in the manufacture of TNT due to incomplete trinitration. They comprise about one percent of the final product[3] and consistently occur in the mix of nitrobodies found in wastewaters from plants that manufacture or load TNT. Spanggord et al.[4] found the DNT isomers to be the dominant components in the condensate wastewater from TNT manufacture at Volunteer Army Ammunition Plant (AAP), Chattanooga TN. 2,4-DNT occurred at an average concentration of 14.7 mg/L and 2,6-DNT at 7.3 mg/L.

The fate of 2,4- and 2,6-DNT in the aqueous environment was reviewed by Spanggord[5]. Volatilization

and sorption to soils and sediments were found to be slight or unimportant factors in altering or sequestering these substances in environmental systems. Photolysis is an important mechanism of transformation of the DNT's, producing such products as dinitro- and aminonitrobenzoic acids, and azoxy compounds.

Biotransformation of 2,4- and 2,6-DNT in natural waters or in the presence of sewage effluents or sludges has been reported in several studies and has been reviewed by Etnier[2] and by Brower and Hartley[6]. Transformation products included aminonitro-toluenes, nitrosonitrotoluenes, and dinitroazoxytoluenes. Transformation of 2,4-DNT by fungal species in pure culture has been reported[7,8]. A suggested scheme for the biotransformation of 2,4-DNT, yielding a variety of nitroaromatic products, is given by McCormick et al.[7].

Spanggord et al.[9] reported that eutrophic surface waters lacking a history of nitrobody pollution contain microorganisms that transform 2,4-DNT in the presence of yeast extract (cometabolism). Testing for the use of 2,4-DNT as a sole carbon and energy source was positive only in water samples from a locality (Waconda Bay, TN) that lies downstream from an AAP. From this source mixed enrichment cultures capable of growing on 100-200 ppm 2,4-DNT were developed, and organisms were

isolated in pure culture that were capable of using the compound as a sole carbon source. The kinetics of 2,4-DNT degradation by a high density population of washed cells of the mixed enrichment culture was studied. Pseudo-first-order rate constants of 1.7 X 10^{-1} and 2.6 X 10^{-1} hr^{-1} were determined, corresponding to a half-life of 0.41 or 0.27 hr. The mean second-order rate constant from three determinations was 4.7 X 10^{-8} ml $cell^{-1}$ hr^{-1}.

The validity of predicting rates of biodegradation in the environment from laboratory determinations has been questioned[10]. In surface waters, xenobiotic compounds are often present in extreme dilution, and this may prevent the development of populations capable of enhanced biodegradation. Rates of biotransformation and biodegradation have been shown to be concentration-dependent for many compounds[10].

The present study was undertaken to confirm and enlarge upon the results of Spanggord[9] by investigating the biodegradation (mineralization) of both 2,4- and 2,6-DNT and comparing the two compounds as to degradation kinetics and the occurrence and increase of degrading populations. The effect of DNT concentration on degradation rate and the development of degrading populations was also studied.

MATERIALS AND METHODS

Water Samples

Samples of surface water were obtained both from the local area (Frederick, MD) and downstream from the Radford Army Ammunition Plant (RAAP) Radford, VA. Collections were made December, 1985 to March, 1986.

Local area samples were taken at the following locations: Carroll Creek at Montevue Lane, Frederick; Monocacy River at Michael's Mill Road, Buckeystown, MD; Tuscarora Creek below the Eastalco Co. aluminum reduction plant, Adamstown, MD; and the Potomac River (Maryland side) at White's Ferry, MD. Stream bottoms were in all cases muddy or silty.

RAAP area samples were obtained from Stroubles Creek and from the New River one-half mile (0.8km) downstream from Stroubles Creek. These sites are located a short distance downstream from the industrial wastewater outflows of the ammunition plant.

All samples were obtained aseptically and were kept cold during transport and maintenance. The samples were collected from the bottom, with some sediments present. Water without sediment was prepared from the samples by allowing to settle for 1 hr and then filtering through two layers of cloth. Biodegradation studies were initiated within 48 hrs of sample collection.

Primary Screening For Biodegradation

Bulk sediments (0.6-1.5 percent v/v) were removed from a portion of each water sample by allowing to settle for 1 hr followed by filtration through two layers of a fine mesh polyester cloth. Biodegradation was tested for in shake flasks containing 200 ml of water sample (without sediments), test chemical at the desired concentration, and buffer (0.2 percent Na_2HPO_4, 0.01 percent $(NH_4)_2SO_4$, pH 7.0). Flasks were incubated for 6 weeks at 25°C.

Aliquots (4.0 ml) were withdrawn throughout the course of the experiment and were assayed for the test chemicals by UV scan (220-360 nm) in a Beckman DU-7 spectrophotometer. Aliquots were centrifuged for 10 min at 10,000 RPM before scanning, and when necessary a hexane extraction step was included. Numbers of total bacteria were determined (in triplicate) by plating serial dilutions on Standard Methods Agar (Difco). Matched control flasks lacked test chemical (DNT) or contained water samples that had been autoclaved for 15 minutes prior to incubation with the appropriate test chemical.

Microbial Enrichment Cultures

To develop mixed enrichment cultures for each of the DNT isomers under study, material from river water

primary screening flasks showing disappearance of DNT was used. Aliquots (5 ml) were used to inoculate shake flasks containing 10 and 20 µg/mL of the appropriate DNT isomer in 95 mL buffered saline medium (BSM) containing trace elements[11]. The flasks were incubated at 25°C, and were monitored for the disappearance of DNT by UV spectrophotometry and gas chromatography. After incubation an aliquot from the highest concentration showing degradation was transferred to flasks containing the same and higher concentrations. The process was repeated stepwise using higher concentrations of the test compounds. When the series of transfers was terminated, the cultures were centrifuged at 16,000 X G, and the pellets were frozen at -70°C in BSM with 5 percent dimethyl sulfoxide.

Kinetic Studies With Enrichment Microorganisms

The rate at which each isomer of DNT could be degraded by the corresponding mixed enrichment culture was determined in pseudo-first order kinetic studies at high bacterial levels. Microorganisms of the mixed enrichment cultures were grown in 2800 mL Fernbach flasks, with shaking, in 600 mL BSM buffer containing 130 ppm of the desired isomer of DNT. When the culture was well grown and the DNT depleted, the culture was centrifuged at 8000 RPM for 10 min and the pellet was washed and resuspended in 100 mL BSM. The cells were

then rested for 5 hrs in a 25°C shaker and were concentrated and resuspended in fresh BSM buffer.

The test compound in BSM was then added, at low final concentration, and the flask was incubated at 25°C with low speed stirring. Samples (2 mL) were removed at zero time and regular intervals and placed in ice. To each sample 3 mL of cold methanol was added. The samples were centrifuged at 5000 RPM for 10 min in the cold to remove cells. The supernatants were stored at -20°C to await analysis by high performance liquid chromatography (HPLC). Samples for bacterial count on Standard Methods agar were taken at time zero and at the end of each run. Pseudo-first and second order rate constants were calculated for each run.

Carbon Dioxide Evolution

The production of CO_2 from unlabeled or ^{14}C-labeled DNT (indicating mineralization) was measured by the method of Gledhill[12] using 5.0 mL 0.5 N KOH in the trap. Labeled CO_2 production was determined by liquid scintillation counting of 1.0 mL aliquots of the KOH in 15 mL Instagel (Packard Instrument Co.) in a Beckman model LS 3801 programmable counter. Gassing for mineralization studies was accomplished with 30 percent oxygen in nitrogen.

Short-term Mineralization Studies

Short-term mineralization studies were conducted as previously described[13]. Mineralization rates of 2,4- and 2,6-DNT were compared by use of the ^{14}C-labelled chemicals (specific activity: 0.16μCi/μg) at concentrations from 1.0 to 15 ppb in triplicate 100 ml surface water samples. Incubation was for 4 hrs at 25^{o}C, after which samples were acidified with 0.2 ml 2N H_2SO_4, and incubated for an additional 6 hrs. $^{14}CO_2$ was taken up on filter papers wetted with 0.15 ml 1N KOH and subjected to scintillation counting. Controls for abiotic radioactive background consisted of identical samples amended with 0.1 percent $HgCl_2$. Mineralization data were corrected to 100 percent trapping efficiencies by acidifying triplicate samples containing 0.1 μCi $NaH^{14}Co_3$. Mineralization velocities were calculated from the data less abiotic background by multiplying the fraction of total labeled substrate mineralized by its concentration and then dividing by the incubation time.

Kinetics Of Microbial Degradation

Pseudo-first-order rate constants ($k_{b'}$) were obtained from regression curves of multiple data points using the following equation:

$$\ln \frac{Co}{Ct} = k_b' t$$

where C_o and C_t are substrate concentrations at time zero and time t; and t is reaction time in minutes. Bacterial counts (B) were determined by plating (duplicate) on Standard Methods Agar and using the mean count for samples taken at the beginning and end of the reaction. Second-order rate constants (k_{b2}) were obtained by dividing the pseudo-first-order rate constant by the bacterial level[11,14].

The half-life of the chemical at 25°C in the presence of a microbial population of 10^6 degraders/ml is estimated from the second-order rate constant by the relation

$$T_{1/2} = \frac{\ln 2}{k_{b2} \cdot B}$$

where B is taken to be 10^6 CFU/mL.

Analytical Methods

The contents of culture flasks (100 mL aliquots) were extracted twice with methylene chloride, evaporated to dryness in a Kuderna-Danish apparatus, and reconstituted in 0.5 mL CH_2Cl_2. The samples were then subjected to thin-layer chromatography (TLC).

Samples (2 mL) from the kinetic studies with enrichment organisms, after addition of 3 mL of

methanol and centrifugation to remove cells, were subjected to HPLC on a Hewlett-Packard HP 1050 system without dilution and without internal standard-ization. Conditions were an isocratic flow of 1.5 mL/min with 70 percent methanol on a Microsorb 25 cm by 4.6 mm C18 column at 25°C. Individual components were identified by their retention times and quantitated by integration of their UV absorbance at 244nM. Calibration curves were prepared by dilution of standards, using six concentrations. Curves with less than 0.98 linearity were rejected.

Chemicals

Unlabeled 2,4- and 2,6-DNT were purchased from Aldrich Chemical Co., Milwaukee, WI. ^{14}C-uniformly-labeled 2,4- and 2,6-DNT were custom-prepared by New England Nuclear Corp., Boston, MA. These were supplied at specific activities of 51.04 mCi/mmol (2,4-DNT) and 28.94 mCi/mmol (2,6-DNT). The labeled 2,4-DNT was diluted with unlabeled compound to yield a stock solution with the same specific activity as the 2,6 isomer.

RESULTS

Screening For Biodegradation

Surface water samples from six sites (see Materials and Methods) were tested for biodegradation

TABLE 1

Incubation of 2,4- and 2,6-DNT with Natural Water Samples

Sample Source	Test Compound	Lag Time	Time to Completion
New River	2,4-DNT	2 da	5-6 da
	2,6-DNT	2-5 da	9-11 da
Stroubles Creek	2,4-DNT	ca.2 da	5-6 da
	2,6-DNT	5 da	ca.44 da

of 20 ppm 2,4-DNT and 20 ppm 2,6-DNT. No degradation of either compound, and no difference between experimental flasks and autoclaved controls was observed with water samples from the four local (Frederick, MD) area sites. Flasks were observed for 27 to 43 days, while total bacterial counts ranged from 1.0×10^4 to 2.2×10^4 CFU/mL.

Water samples from either of the two sites downstream from Radford AAP completely degraded 20 ppm of either DNT isomer. This did not occur in control samples, in which autoclaved water was used. Some lag time prior to significant disappearance of DNT was noted, and this was considerably longer when 2,6-DNT was tested in the presence of water from the Stroubles Creek site (Table 1).

Total CFU was 9.7×10^4/mL in the New River sample and 9.5×10^4/mL in the Stroubles Creek sample. Most

TABLE 2

Population of DNT Utilizers (MPN) in Natural Water Samples before and after Incubation with DNT

Sample Source	2,4-DNT		2,6-DNT	
	Before	After	Before	After
New River	2.3×10^{1}	3.5×10^{4}	7.8×10^{-1}	2.4×10^{2}
Stroubles Creek	2.3×10^{0}	n.d.	n.d.	n.d.

Probable Numbers (MPN) were determined for 2,4-DNT utilizers and 2,6-DNT utilizers before and after incubation with the relevant DNT (Table 2).

Comparative Degradation of 2,4- and 2,6-DNT and Concentration Effect

Degradation of 2,4- and 2,6-DNT, at concentrations from 0.004 ppm to 10.0 ppm was tested in 80 percent New River Water (80 percent sample water plus 20 percent Solution A). ^{14}C-DNT (U-ring labeled) was present at 0.16 μCi/250 ml flask. Control flasks contained autoclaved New River water. Trapped carbon dioxide was counted in the scintillation counter. Results are graphed in Figures 1 and 2, showing percent of input DNT converted to CO_2. Maximum rates of mineralization are given in Table 3. No CO_2 was detected in autoclaved control flasks.

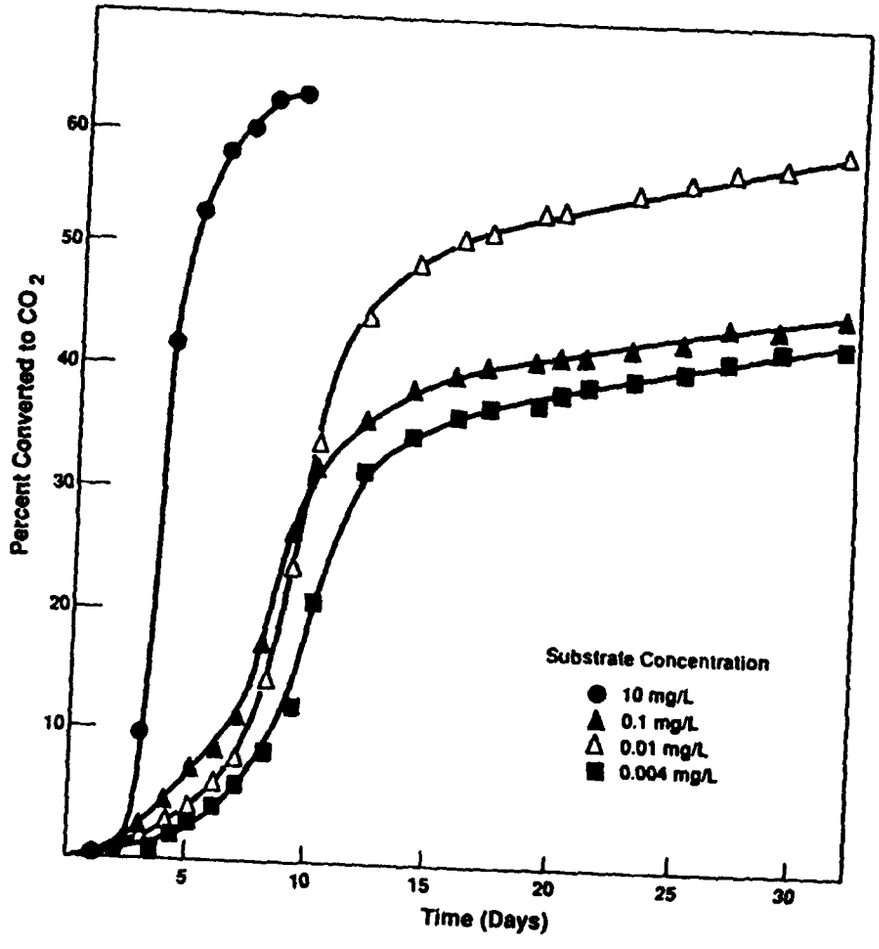

FIG. 1. Formation of Carbon Dioxide from 2,4-DNT in New River Water

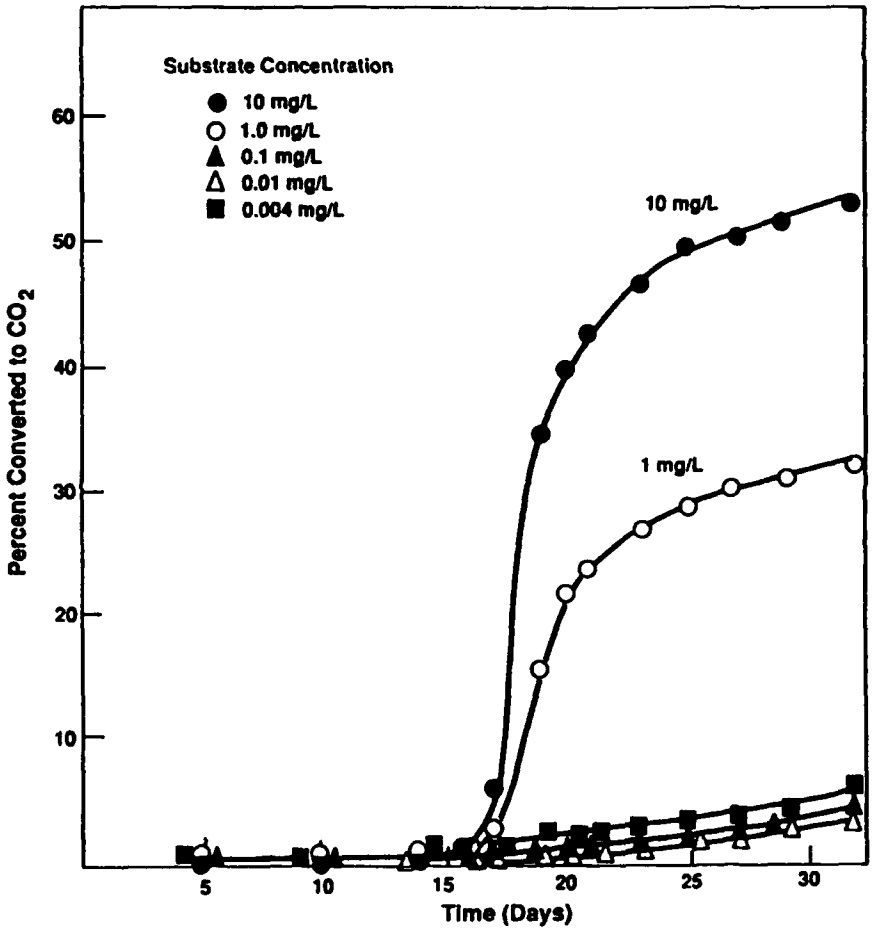

FIG. 2. Formation of Carbon Dioxide from 2,6-DNT in New River Water

TABLE 3

Maximum Rates of Mineralization of DNT in Natural Water Samples

Concentration (ppm)	percent/day	mg/L/day	*Percent degraded
2,4-DNT			
10.0	32.3	3.23	64
0.1	10.7	0.0107	47
0.01	10.9	0.0011	60
0.004	10.4	0.0004	45
2,6-DNT			
10.0	14.5	1.45	55
1.0	5.45	0.055	33
0.1	0.2	0.0002	5
0.01	---	0.00002	4
0.004	---	0.000008	6

*No CO_2 was recovered from control (autoclaved) flasks.

Both isomers show clear evidence of mineralization to CO_2. With both, the rate of mineralization was observed to be concentration-dependent, increasing with increasing concentration. Degradation of 2,4-DNT began at 2 to 3 days incubation, at all concentrations tested, and yielded from 45 to 65 percent of the added label as CO_2. Degradation of 2,6-DNT was not initiated until about 17 days of incubation and did not occur to any significant degree at substrate concentrations below 1.0 ppm

Most Probable Number (MPN) results show 2,4-DNT degraders to be present initially at far higher concentrations than 2,6-DNT degraders: 1.1×10^3/ml vs. 7.9×10^0/ml. In the presence of the corresponding DNT

isomer at 10 ppm, 2,4-DNT degraders increased 155-fold to 1.8 X 10^5/ml and 2,6-DNT degraders increased 62-fold to 4.9 X 10^2/ml. At substrate concentrations of 0.1 ppm, no increase in degrader biomass was seen. In addition, degrading populations were observed to undergo viability losses at substrate concentrations below 0.1 ppm.

Thus it appears that the 2,4 isomer can be degraded faster in natural waters than 2,6-DNT. Furthermore, a degrading population does not develop at very low concentrations of the 2,6 isomer.

Short-term Degradation of 2,4- and 2,6-DNT in Natural Water Samples

Mineralization of DNT was determined in New River water containing various concentrations of ^{14}C-2,4-DNT or ^{14}C-2,6-DNT from 1.0 ppb to 15 ppb (specific activity, 0.16 μCi/μg). Rates of degradation were determined by $^{14}CO_2$ recovered and calculated as ng DNT degraded per 0.1 L per hour. Rates are shown as a function of substrate concentration in Figure 3. Control flasks contained 0.1 percent $HgCl_2$.

The rates of substrate utilization are far greater for the 2,4 isomer than for the 2,6 isomer. This is consistent with the far greater population density of 2,4-DNT degraders present in the water: 1.08 X 10^3/ml vs. 0.04/ml 2,6-DNT degraders. In the case of 2,4-DNT

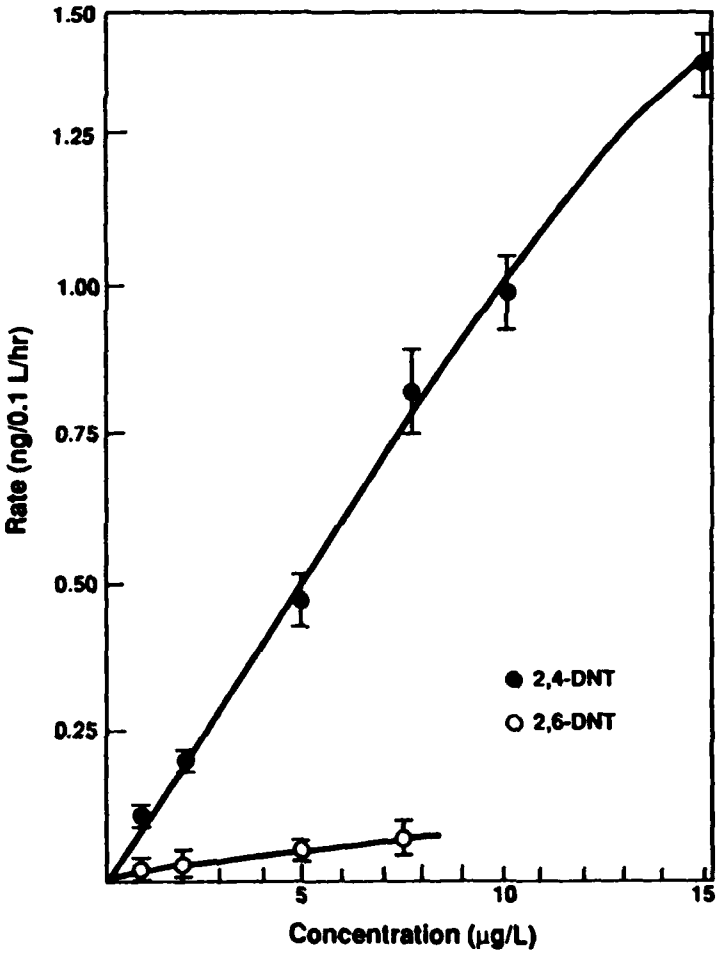

FIG. 3. Rate of Mineralization of Dinitrotoluene in New River Water as a Function of Substrate Concentration

utilization, the uppermost three points on the curve tend toward saturation. The data do not support the calculation of $1/V_{max}$ values, although in the case of 2,4-DNT this appears to be about 40 to 50 ng L^{-1} hr^{-1}.

Microbiological Enrichments

Material from primary screening cultures was passed sequentially through increasing levels of 2,4- or 2,6-DNT, with degradation of the test compound and visible turbidity noted in each case up to the level of 130 ppm test compound. Attempts to pass the microbes to DNT levels higher than this did not succeed.

To study the growth and activity of the 2,4-DNT and 2,6-DNT enriched cultures on 100 µg/ml (100ppm) of the corresponding test compound, the 130 ppm cultures were pelleted and resuspended three times in BSM, with 2 hrs holding time following the second resuspension. Experimental flasks and control flasks with autoclaved cells contained ^{14}C-DNT, 1.0 µCi/flask. Other control flasks contained no test compound. The results are shown in Figure 4. The bacterial population in the 2,4-DNT experimental flask was 1.4 X 10^6/ml at the outset and increased 53-fold at 42 hrs (insert). In the 2,6-DNT flask, the beginning population was 4.9 X 10^5/ml and increased 140-fold at 96 hrs. The 2,4-DNT mixed culture grew at a substantially faster rate than the 2,6-DNT culture.

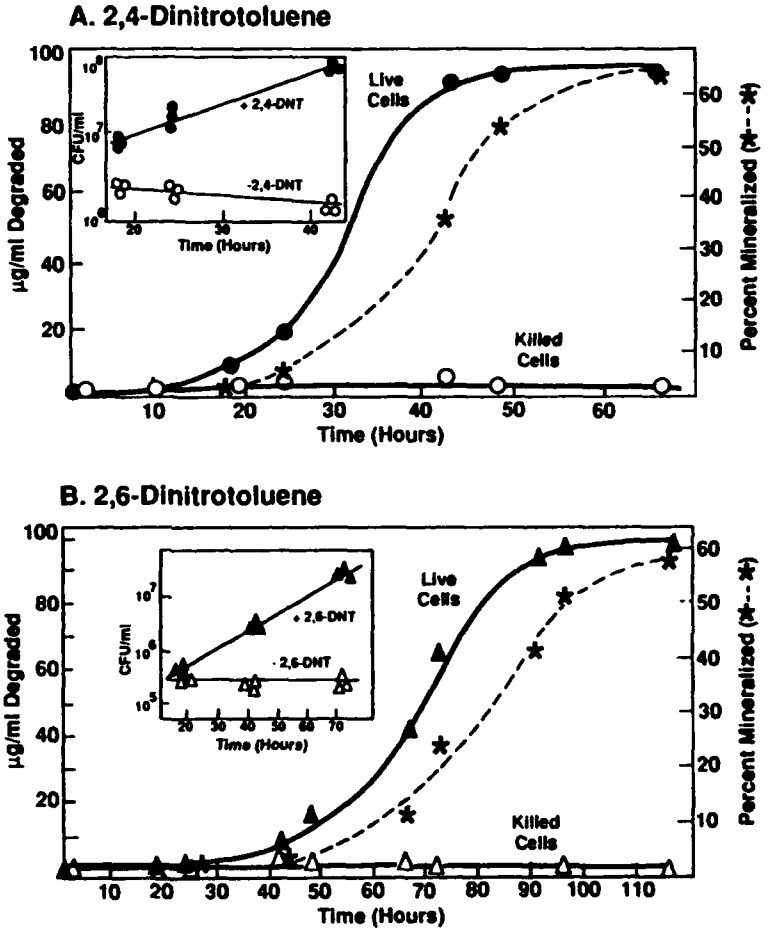

FIG. 4. Mineralization of DNT by Mixed Enrichment Cultures of New River Organisms. Insert: Growth of Bacterial Populations

It is clear that both isomers of DNT can serve as sole carbon and energy source for bacterial growth. Recoveries of input radioactivity as $^{14}CO_2$ were about 60 percent, showing clearly that the ring structure of both compounds was broken and that most if not all of the material was mineralized. Disappearance of DNT was close to 100 percent in both cases. Cell yields were 6.8×10^5 to 7.3×10^5 CFU/μg input DNT. Degradation of test compound took place much faster in the case of 2,4-DNT than in the 2,6-DNT test.

The mixed culture active on 2,6-DNT included at least four bacterial types distinguishable by colony morphology, and this assemblage did not change noticeably on repeated passage in 2,6-DNT medium. This mixed culture was able to grow in 2,4-DNT medium, with some evidence of a process of acclimation. The reverse did not occur: growth of the 2,4-DNT mixed culture in 2,6-DNT medium. Derivation of pure strains using the pertinent DNT isomer as sole carbon source was successful with 2,4-DNT but not 2,6-DNT.

Kinetics of Transformation of 2,4- and 2,6-Dinitrotoluene

The rate at which each isomer of DNT could be degraded by the corresponding mixed enrichment culture was determined in pseudo-first order kinetic studies at high bacterial levels, as described under Materials and

Methods. The cells were washed, concentrated, and resuspended in fresh BSM buffer. They were then incubated in shake flasks at 25°C in the presence of the appropriate isomer of DNT at fairly low concentration. The disappearance of DNT was followed by HPLC analysis of samples taken at regular intervals, and bacterial levels were enumerated. Pseudo-first and second order rate constants were calculated for each run as described in Materials and Methods.

Results of two typical experiments with 2,4-DNT at a starting concentration of 6.4-8.5 μg/ml are shown in Figure 5. The rate of DNT disappearance is seen to increase during the initial part of each curve, following which it is approximately exponential (first-order) for the remainder of the test. The straight line is the calculated regression for the section of the curve judged to be exponential, and its slope was used as an estimate of the pseudo-first order rate constant.

The results of eight such determinations for the 2,4-DNT mixed culture growing on various concentrations of 2,4-DNT are shown in Table 4. Total bacteria did not change significantly during the experiment. Bacterial concentrations measured at the beginning and end of the run were averaged and the result used to calculate the second order rate constant. The mean

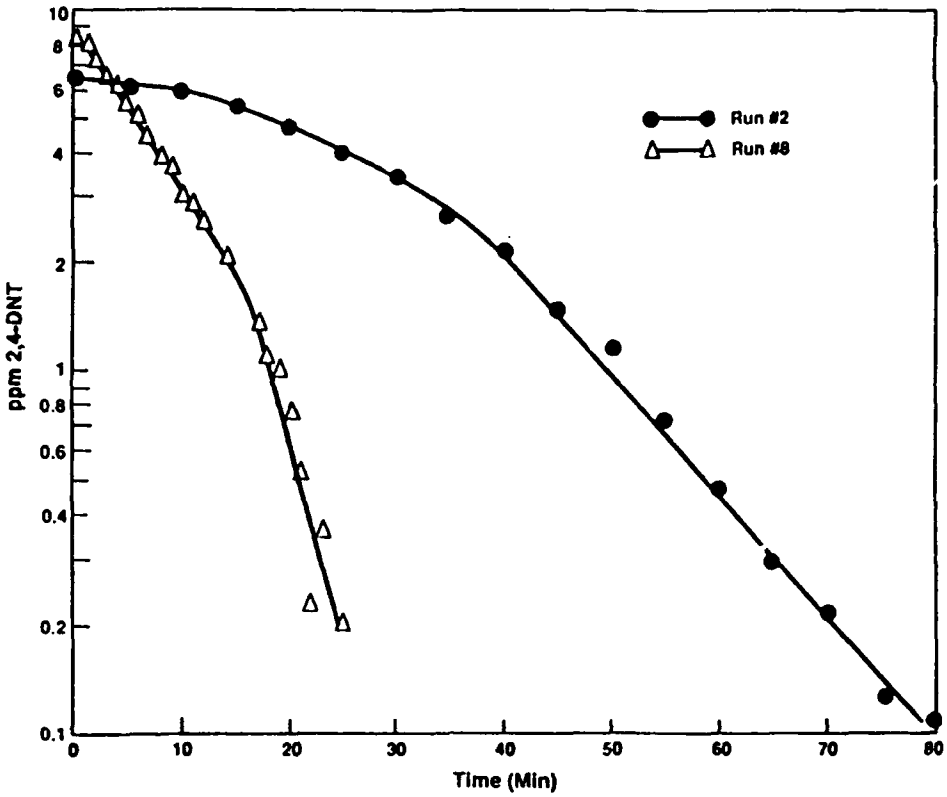

FIG. 5. Uptake of 2,4-DNT by Enrichment Microorganisms

TABLE 4

Rate Constants for 2,4-DNT Uptake by 2,4-DNT Mixed Enrichment Culture

Run	DNT Conc. (μg/ml)	Bact Conc. (CFU/ml)	R^2	(n)	Rate Constant: Pseudo-first (min^{-1})	Rate Constant: Second Order (ml $cell^{-1}$ min^{-1})
1	9.0	2×10^8			0.071	3.6×10^{-10}
2	9.0	2×10^8	0.860	(10)	0.075	3.8×10^{-10}
3	9.0	2×10^8			0.043	2.2×10^{-10}
4	9.0	1×10^8	0.969	(9)	0.048	4.8×10^{-10}
5	1.0	2.9×10^8	0.946	(10)	0.121	4.2×10^{-10}
6	6.0	2.8×10^8			0.084	3.0×10^{-10}
7	4.5	2×10^8	0.942	(21)	0.108	5.4×10^{-10}
8	11.1	4.4×10^8	0.968	(10)	0.190	4.3×10^{-10}
Mean						3.89×10^{-10}

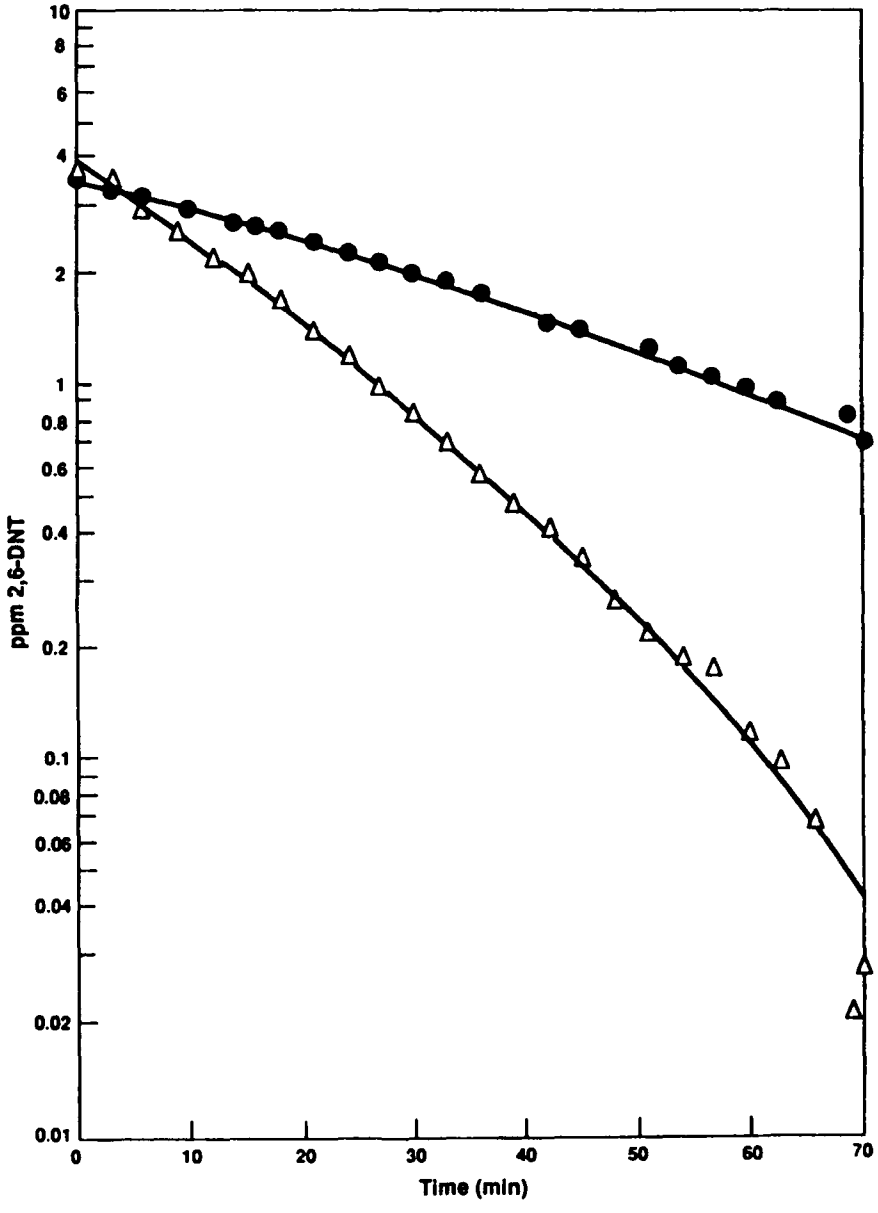

FIG. 6. Uptake of 2,6-DNT by Enrichment Microorganisms

TABLE 5

Rate Constants for 2,6-DNT Uptake by 2,6-DNT Mixed Enrichment Culture

Run	DNT Conc. (μg/ml)	Bact Conc. (CFU/ml)	R^2	Rate Constant Pseudo-first (n)	Rate Constant Pseudo-first (min^{-1})	Rate Constant Second Order (ml $cell^{-1}$ min^{-1})
1	12.0	2.6×10^7	0.939	(27)	0.035	1.37×10^{-9}
2	10.2	6.9×10^7	0.885	(11)	0.094	1.36×10^{-9}
3	3.6	2.0×10^7	0.977	(26)	0.023	1.14×10^{-9}
4	3.8	7.1×10^7	0.899	(18)	0.057	8.0×10^{-10}
5	20.0	1.2×10^8	0.895	(31)	0.035	2.91×10^{-10}
ean						9.92×10^{-10}

value for the disappearance of 2,4-DNT in the presence of enrichment microorganisms was 3.89×10^{-10} ml $cell^{-1}$ min^{-1}.

Results of two typical experiments with 2,6-DNT at a starting concentration of 3.6-3.8 μg/ml are shown in Figure 6. As with the 2,4- isomer, the rate of DNT disappearance is approximately exponential, i.e. pseudo-first order, over most of the curve.

Results of five determinations for the 2,6-DNT mixed culture growing on various concentrations of 2,6-DNT are shown in Table 5. The mean value for the disappearance of the test compound in the presence of 2,6-DNT was 9.92×10^{-10} ml $cell^{-1}$ min^{-1}.

DISCUSSION

The dinitro-substituted toluenes and benzenes seem far more amenable to degradation, i.e. to ring cleavage and mineralization, than the corresponding trinitro compounds. Thus Mitchell et al.[15] found that 1,3-dinitrobenzene (DNB) was readily mineralized by microorganisms occurring in surface waters while 1,3,5-trinitrobenzene and 3,5-dinitroaniline were not. Spanggord et al.[9] reported that 2,4-DNT was readily degraded but not 2,4,6-TNT. All of these compounds undergo microbially mediated transformation to related compounds without degradation through ring cleavage. The present work confirms the ready degradability of 2,4-DNT and extends the finding to include the 2,6 isomer. Both are mineralized in the presence of competent microorganisms and support the growth of the latter. In contrast to the earlier results[15] with DNB, however, neither degradation nor biotransformation of the DNT's was observed in water samples from sites with no history of pollution by munition compounds. This may be explained by the adaptation of microorganims over time in the vicinity of the AAP to discharges containing these compounds.

In tests for the disappearance of 2,4- and 2,6-DNT in water samples from sites downstream from

Radford (VA) AAP, the 2,4 isomer was characterized by a much higher disappearance rate and much shorter lag time. This was associated with a much larger population of organisms capable of acting on the compound, as shown by Most Probable Number determinations. While acting on these compounds, the population of biodegraders increased many-fold. At 10 ppm DNT, this increase was 155-fold for the 2,4 isomer and 62-fold for 2,6-DNT.

The concentration of the test compound was an important determinant of biodegradation. The rate of degradation and percent of compound mineralized increased greatly at the higher concentrations tested. Biodegrader populations were greatly influenced by initial DNT concentration. At the higher concentrations, 10 ppm or above, degrader populations increased; while at initial concentrations of 1.0 ppm or below increase was not detected and some decrease (dieoff) was seen. This suggests that microbial degradation may play only a minor role in the fate of DNT in waterways when present in low concentrations.

Studies with mixed enrichment cultures developed from New River water samples indicate that both DNT isomers under study can be used as sole sources of carbon and energy by microorganisms (Figure 4). The observation that ca. 60-65 percent of input DNT carbon

was converted to CO_2 suggests that all or nearly all was biodegraded, since a substantial fraction of the substrate may have been converted into biomass. This is substantiated by the increase in microbial populations observed. It is likely that all parts of the nitrotoluene structure are susceptible to metabolic breakdown by microorganisms.

Kinetic studies on 2,4- and 2,6-DNT conducted with high levels of the corresponding mixed enrichment culture indicated that the second order rate constant for the 2,4 isomer, based on eight runs, was 3.9 X 10^{-10} ml $cell^{-1}$ min^{-1}. This compares to an estimate of 7.8 X 10^{-10} ml $cell^{-1}$ min^{-1} found by Spanggord et al.[9] for a mixed microbial culture from Waconda Bay, TN, situated downstream from the Volunteer AAP. From the rate constant, found in the present study a half-life of 29.7 hrs can be estimated for this isomer in the presence of 10^6 cells/ml at 25°C. Similarly, the mean second-order rate constant for 2,6-DNT uptake was 9.9 X 10^{-10} ml $cell^{-1}$ min^{-1}. The corresponding estimated half-life is 11.6 hrs.

This would seem to contradict the result seen in the natural water samples, wherein the rates of both disappearance and mineralization of 2,4-DNT were several times greater than those for the 2,6 isomer. The difference may lie in the fact that in the natural

water samples significant utilization of the nitroaromatic depends on extensive population growth. Thus slow or delayed development of the degrading population is reflected in slow or delayed substrate utilization. The high density populations used in the mixed culture kinetic experiments, in contrast, experience little growth during the experiment, and the observed utilization rates are those of established, rested populations. The intrinsic rates of uptake of DNT per competent cell may differ only slightly between the two isomers studied.

SUMMARY AND CONCLUSIONS

1. The microbially-mediated degradation of 2,4- and 2,6-DNT was complete or nearly so in surface water samples taken downstream from Radford AAP. Degradation was not observed in samples from the Frederick (Maryland) area.

2. Compared to the 2,6 isomer, 2,4-DNT was degraded at a much higher rate in surface water samples, with a far shorter lag time, and the corresponding population of degrading organisms was far larger.

3. Both substrates serve as sole sources of carbon and energy for microbial growth, with up to 60 percent of substrate carbon appearing as CO_2.

4. Degradation rates, in percent degraded per day, increased with increasing substrate concentration. At very low substrate concentrations, degrader populations did not increase.

5. Mixed enrichment cultures were developed for each isomer. Maximal substrate concentrations tolerated were 130 mg/L. Cell yields were about 7 X 10^5 CFU/μg input DNT.

6. From enrichment cultures, pure strains using 2,4-DNT were isolated.

7. Degradation of substrate by high concentration of enrichment organisms approximated first-order kinetics. For 2,4-DNT, mean second-order rate constant was 3.9 X 10^{-10} ml $cell^{-1}$ min^{-1}, while for 2,6-DNT this was 9.9 X 10^{-10} ml $cell^{-1}$ min^{-1}.

8. The slower degradation rates observed for the 2,6 isomer in natural surface waters may be related to the fact that in the latter significant utilzation of the nitroaromatic depends on extensive growth of an initially small degrading population.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. Helen T. Hargett and Mr. Robert G. Routier for technical assistance and Mr. Alan B. Rosencrance for technical support in chemistry.

DISCLAIMER

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REFERENCES

1. Beard RR, Noe JT. Aromatic nitro and amino compounds. In: Clayton GD, Clayton FE. eds. Patty's Industrial Hygiene and Toxicology, Vol. 2A. New York: John Wiley & Sons 1981 (3rd ed.)

2. Etnier EL. Water Quality Criteria for 2,4-Dinitrotoluene and 2,6-Dinitrotoluene. Final Report. AD-A188 713. Oak Ridge National Laboratory, Oak Ridge, TN, 1987.

3. Ryon MG, Pal BC, Talmage SS, Ross RH. Database Assessment of Health and Environmental Effects of Munition Production Waste Products. Report ORNL-6018, Oak Ridge National Laboratory, Oak Ridge, TN, 1984.

4. Spanggord RJ, Gibson BW, Keck RG, Newell GW. Mammalian Toxicological Evaluation of TNT Wastewaters, Vol. 1, Chemistry Studies. Annual Report. SRI International, Menlo Park, CA, 1978.

5. Spanggord RJ, Mill T, Chou T-W, Mabey WR, Smith JH, Lee S. Environmental Fate Studies on Certain Munition Wastewater Constituents. Phase I - Literature Review. AD A082 372. SRI International, Menlo Park, CA, 1980.

6. Brower ME, Hartley WR. Health Advisory for 2,4- and 2,6-Dinitrotoluene (DNT). US Environmental Protection Agency, Criteria and Standards Division, Washington, DC, in press.

7. McCormick NG, Cornell JH, Kaplan AM. Identification of Biotransformation Products from 2,4-Dinitrotoluene. Appl. Environ. Microbiol. 1978; 35(5): 945-948.

8. Parrish FW. Fungal Transformation of 2,4-Dinitrotoluene and 2,4,6-Trinitrotoluene. Appl. Environ. Microbiol. 1977; 34(2): 232-233.

9. Spanggord RJ, Mill T., Chou T-W, Mabey WR, Smith JH, Lee S. Environmental Fate Studies on Certain Munition Wastewater Constituents - Phase II - Laboratory Studies. AD A099 256. SRI International, Menlo Park, CA, 1980

10. Alexander M. Biodegradation of Organic Chemicals. Environ. Sci. Technol. 1985; 18: 106-111.

11. Smith JH, Mabey WR, Bohonos N, Holt BR, Lee SS, Chou T-W, Bomberger DC, Mill T. Environmental Pathways of Selected Chemicals in Freshwater Systems. Part I, Background and Experimental Procedures. EPA/600/01. Final Report to US Environmental Protection Agency, SRI International, Menlo Park, CA, 1977.

12. Gledhill WE. Screening Test for Assessment of Ultimate Biodegradability: Linear Alkylbenzene Sulfonates. Appl. Microbiol. 1977; 30:922-929.

13. Mitchell WR. Biodegradation of Guanidinium by Aquatic Organisms. AD A164 728. Technical Report 8506, US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD, 1985.

14. Paris DF, Steen WC, Baughman GL, Barnett JT. Second-order Model to Predict Microbial Degradation of Organic Compounds in Natural Waters. Appl. Environ. Microbiol. 1981; 41: 603-609.

15. Mitchell WR, Dennis WH, Burrows EP. Microbial Interactions with Several Munitions Compounds: 1,3-Dinitrobenzene, 1,3,5-Trinitrobenzene, and 3,5-Dinitroaniline. AD A116 651. Technical Report 8201, US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, MD, 1982.

Date Received: 08/30/91
Date Accepted: 10/07/91